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14. ABSTRACT

The purpose of this research is to gain a better understanding of the biochemical pathways and molecular targets for the selective induction of apoptosis signaling and execution of prostate cancer (PCa) cells by methyl selenium. We hypothesized that methyl selenium inhibits PI3K-AKT survival pathway leading to the activation of caspase-dependent apoptosis execution in PCa cells.

The specific aim for the no-cost extension period is to define early signaling mechanisms induced by methylselenium for apoptosis and how and through what targets AKT regulates death signaling. We have identified BCl-xL, FLIP and survivin as early targets affected by MSeA. This was designed to follow up on a novel lead for using selenium as a chemosensitizer for cancer therapeutic drug-induced apoptosis in androgen independent PCa cells.

Work accomplished: We have tested the functional significance of MSeA-induced down regulation of Bcl-xL and survivin through the ectopic expression of these two genes in DU145 cells and tested their impact on apoptosis induced by taxol. The results have been accepted for publication in Clinical Cancer Res. Re-print is appended for this addendum report.

15. SUBJECT TERMS
Selenium, methylselenol, prostate cancer chemoprevention, apoptosis

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Report Body

Addendum Report of accomplishment during approved no-cost extension period (Jan 2007-Jan 2008)

1. Specific aim for the no-cost extension period: To define early signaling mechanisms induced by methylselenium for apoptosis and how and through what targets AKT regulates death signaling. We have identified BCl-xL, FLIP and survivin as early targets affected by MSeA.

2. Work accomplished:

We have been able to test the functional significance of MSeA-induced down regulation of Bcl-xL and survivin through the ectopic expression (stable and transient transfection of expression plasmids) of these two genes in DU145 cells and tested their impact on apoptosis induced by taxol. The results have been accepted for publication in *Clinical Cancer Res*. The essence of this paper is summarized in the following:

ABSTRACT

PURPOSE: Our previous work has shown that methylseleninic acid (MSeA) sensitized hormone refractory prostate cancer (HRPCa) cells to apoptosis induced by paclitaxel (taxol) through enhancing multiple caspases. This study aimed to: 1) determine the general applicability of the sensitization effect for taxane drugs *in vitro*; 2) establish the enhancement of paclitaxel efficacy by MSeA *in vivo*; and 3) investigate Bcl-XL and survivin as molecular targets of MSeA to augment apoptosis.

EXPERIMENTAL DESIGN: DU145 and PC-3 HRPCa cell lines were used to evaluate the *in vitro* apoptosis effects of paclitaxel, docetaxel and their combination with MSeA and the molecular mechanisms. DU145 xenograft growth in athymic nude mice was used to evaluate the *in vivo* efficacy of paclitaxel and its combination with MSeA. The tumor samples were used to examine Bcl-XL and survivin protein abundance.

RESULTS: MSeA combination with paclitaxel or docetaxel exerted a greater than additive apoptosis effect on DU145 and PC-3 cells.   In nude mice, paclitaxel and MSeA combination inhibited growth of DU145 subcutaneous xenograft with the equivalent efficacy of a 4-time higher dose of paclitaxel alone.   MSeA decreased the basal and paclitaxel-induced expression of Bcl-XL and survivin *in vitro* and *in vivo*. Ectopic expression of Bcl-XL or survivin attenuated MSeA/paclitaxel-induced apoptosis.

CONCLUSIONS: MSeA enhanced the efficacy of paclitaxel against HRPCa *in vitro* and *in vivo*, at least in part by down-regulating the basal and paclitaxel-induced expression of both Bcl-XL and survivin to increase caspase-mediated apoptosis. MSeA may be a novel agent to improve taxane combination therapy.

Acceptance of manuscript describing mechanistic investigation of potential protein targets to mediate efficacy enhancement by *Clin Cancer Res* for publication sara.borden@aacr.org Fri Nov 16 13:51:13 2007
To: jlu@hi.umn.edu
Subject: Decision Rendered: CCR-07-4037R

Dear Dr. Lu:
I am pleased to inform you that your manuscript titled **Methylseleninic acid enhances taxane drug efficacy against human prostate cancer and down regulates anti-apoptotic proteins Bcl-XL and survivin, CCR-07-4037R**, has been accepted for publication in CLINICAL CANCER RESEARCH. You will receive electronic page proofs via e-mail within approximately 8 weeks. If you expect your contact information to change within that time, please contact the Editorial Office immediately either by e-mail to ccr@aacr.org, or by fax to (215)440-9354.
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3. Reportable outcomes

1) Hongbo Hu, Guang-xun Li, Lei Wang, Jennifer Watts, Gerald F Combs, Jr, and Junxuan Lü. Methylseleninic acid enhances taxane drug efficacy against human prostate cancer and down regulates anti-apoptotic proteins Bcl-XL and survivin. *Clinical Cancer Res*. 2008. In Press (included as Appendix (galley proof).

2) A revised grant application based on the data generated from work supported by DOD and NCI has been re-submitted for funding consideration to fully develop the therapeutic potential of methyl selenium with taxane drugs for prostate cancer. (1R01CA129345-01A1 Proposal Title: Methyl Selenium as Chemoenhancer of Prostate Cancer Chemotherapy. To be reviewed Jan 31, 2008)

Methylseleninic Acid Enhances Taxane Drug Efficacy Against Human Prostate Cancer and Down-Regulates Antiapoptotic Proteins Bcl-XL and Survivin

Hongbo Hu,[1] Guang-xun Li,[1] Lei Wang,[1] Jennifer Watts,[2] Gerald F. Combs, Jr.,[2] and Junxuan Lü[1]

Abstract

Purpose: Our previous work has shown that methylseleninic acid (MSeA) sensitized hormone refractory prostate cancer (HRPCa) cells to apoptosis induced by paclitaxel (Taxol) through enhancing multiple caspases. This study aimed to (*a*) determine the general applicability of the sensitization effect for taxane drugs *in vitro*, (*b*) establish the enhancement of paclitaxel efficacy by MSeA *in vivo*, and (*c*) investigate Bcl-XL and survivin as molecular targets of MSeA to augment apoptosis.

Experimental design: DU145 and PC-3 HRPCa cell lines were used to evaluate the *in vitro* apoptosis effects of paclitaxel, docetaxel and their combination with MSeA, and the molecular mechanisms. DU145 xenograft growth in athymic nude mice was used to evaluate the *in vivo* efficacy of paclitaxel and its combination with MSeA. The tumor samples were used to examine Bcl-XL and survivin protein abundance.

Results: MSeA combination with paclitaxel or docetaxel exerted a greater than additive apoptosis effect on DU145 and PC-3 cells. In nude mice, paclitaxel and MSeA combination inhibited growth of DU145 subcutaneous xenograft with the equivalent efficacy of a four-time higher dose of paclitaxel alone. MSeA decreased the basal and paclitaxel-induced expression of Bcl-XL and survivin *in vitro* and *in vivo*. Ectopic expression of Bcl-XL or survivin attenuated MSeA/paclitaxel-induced apoptosis.

Conclusions: MSeA enhanced the efficacy of paclitaxel against HRPCa *in vitro* and *in vivo*, at least in part, by down-regulating the basal and paclitaxel-induced expression of both Bcl-XL and survivin to increase caspase-mediated apoptosis. MSeA may be a novel agent to improve taxane combination therapy.

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Prostate cancer is a serious threat to the health and quality of life of American men and is the second leading cause of cancer-related death. Improvement in the efficacy of chemotherapy is urgently needed for patients with metastatic hormone refractory prostate cancer (HRPCa; ref. 1). The taxane drug docetaxel is the only Food and Drug Administration–approved chemotherapeutic agent for clinical treatment of advanced HRPCa. Two landmark studies (refs. 2, 3; Southwest Oncology Group 9916 and TAX 327) provided the key support for this indication by demonstrating a modest survival advantage in HRPCa patients by using docetaxel-based combination chemotherapy.

These studies supported taxane-based therapy as an attractive platform to develop new combinational regimens, which remain a high priority for many ongoing studies. A better understanding of the mechanisms of taxane resistance could provide a more rational strategy for the development of more effective taxane-based therapeutic combinations.

Taxane drugs disrupt microtubule dynamics through direct binding to the β-subunit of tubulin, leading to mitotic arrest and eventual apoptosis (4). Taxanes are susceptible to several mechanisms of drug resistance. The first is the classic multidrug resistance gene product P-glycoprotein to pump the drugs out of the cells (5). The second is related to microtubule alterations, including mutations that result in changes in either microtubule dynamics or drug binding (6, 7). The third and more recently recognized resistance mechanism is related to the activation of prosurvival pathways by taxane drugs. These include an increased expression of intracellular inhibitors of apoptosis proteins, such as survivin (8) and c-AIP2 (9), and the mitochondria protective antiapoptosis protein Bcl-XL (10, 11), and activation of the nuclear factor-κB pathway (11, 12). Increasing evidence suggests that elevated expression of Bcl-XL (13–19) and survivin (20–24) confers resistance to apoptosis induced by taxanes and other anticancer drugs.

It has been well-established that drug-induced apoptosis in most cancer cells is mediated through the mitochondrial (intrinsic) pathway and/or death receptor (extrinsic) pathway

for caspase activation (25). One of the central control steps for apoptosis induction through the mitochondrial pathway is disruption of mitochondrial membrane potential, leading to the release of cytochrome *c* and caspase-9 activation. This critical step is controlled and mediated by the Bcl-2 family proteins (26). Members of the Bcl-2 family fall into three different classes based on the conservation of Bcl-2 homology domains (BH1-4) and functions: multidomain prosurvival proteins (Bcl-2, Bcl-XL, and Mcl-1), multidomain proapoptotic proteins (Bax and Bak), and BH3-only proapoptotic proteins (Bad, Bid, Bim, PUMA, NOXA, and Bik). The activity of caspases-3, -7, and -9 is often further regulated by survivin and other inhibitors of apoptosis proteins (27). It has been shown that Bcl-XL and survivin are highly expressed in androgen-independent prostate cancer cells (28–30). Therefore, compounds that can down-regulate the basal and taxane-induced expression of Bcl-XL and/or survivin may serve as sensitizers for taxane chemotherapy.

Converging data from epidemiologic, ecological, and clinical studies have implicated selenium as an effective chemopreventive agent particularly for prostate, colon, and lung cancers (31). The chemopreventive potential of selenium compounds is being validated in two ongoing clinical trials for prostate and lung cancer, respectively, in North America (32, 33). Recent studies, including our own, have brought a renewed interest in the therapeutic potential of selenium as an enhancer of existing treatment modalities (34–36). We have shown that methylseleninic acid (MSeA), a synthetic selenium compound that is considered an immediate precursor to the *in vivo* active anticancer selenium metabolite methylselenol, greatly sensitized HRPCa cells to undergo apoptosis induced by paclitaxel (Taxol), the topoisomerase I inhibitor SN-38 and the topoisomerase II inhibitor etoposide, through enhancing multiple caspases (34). However, the mechanisms of the enhancement action remain to be clarified, and the *in vivo* clinical translation potential of the combination treatment for HRPCa has not been established in an animal model.

The objectives of the present study were (*a*) to determine the general applicability of the sensitization effect of MSeA on taxane drugs (paclitaxel versus docetaxel), (*b*) to determine the *in vivo* efficacy of combining paclitaxel with MSeA against DU145 HRPCa xenograft growth in an athymic nude mouse model, and (*c*) to investigate Bcl-XL and survivin as potential molecular targets of MSeA to augment caspase activation and apoptosis.

Materials and Methods

Chemicals and reagents. The MSeA (CH_3SeO_2H) preparation used for the cell culture work was as previously described (37, 38). The MSeA used for animal experiment was purchased from Sigma-Aldrich Co. (purity, 95%). Paclitaxel and docetaxel used for the *in vitro* studies and an antibody for β-actin were also purchased from Sigma-Aldrich Co. The general caspase inhibitor zVADfmk was purchased from MP-Biomedicals, Inc. Antibodies specific for Bcl-XL, survivin, cleaved poly(ADP-ribose)polymerase (PARP; p89), and cleaved caspases-3 were purchased from Cell Signaling Technology. The proteasomal inhibitor MG-132 was purchased from CalBiochem. The injectable paclitaxel solution for animal studies was provided by the Outside Investigator Program of the Bristol-Myers Squibb Co.

Cell culture and treatments. DU145 and PC3 cell lines were obtained from the American Type Culture Collection. DU145 cells were grown in MEM supplemented with 10% fetal bovine serum without antibiotics. PC-3 cells were grown in F-12K medium with 10% fetal bovine serum without antibiotics. DU145 stable transfectant cells with pIRES-neo-Bcl-xL or the pIRES-neo control plasmid (generously provided by Dr. Peter Daniel, Humboldt University, Germany) were grown in DMEM supplemented with 10% fetal bovine serum. Forty-eight hours after plating, when cells were 50% to 60% confluent, the medium was changed before starting the treatment with MSeA or the other agents. To standardize all MSeA/drug exposure conditions, cells were bathed in culture medium at a volume to surface area ratio of 0.2 mL per cm^2 (e.g., 15 mL for a T75 flask and 5 mL for a T25 flask). For the experiment in which the proteasomal inhibitor MG132 was used, the cells were exposed to the drug for 1 h before initiating treatment with MSeA. For the experiments in which the general caspase inhibitor was used, the inhibitor and MSeA were given to the cells at the same time. DMSO (2 μL/mL or less) was added as a vehicle solvent to the control cells. This concentration of DMSO did not cause any adverse morphologic response.

Apoptosis evaluation. Apoptosis was assessed by multiple methods. The first was Annexin V staining of externalized phosphatidylserine in apoptotic cells by flow cytometry using the Annexin V/FITC Staining kit from MBL International, Inc. The second was a cell death detection ELISA kit purchased from Roche Diagnostics Corporation, which we have used in previous studies (34). The third method was immunoblot analysis of PARP cleavage, as described previously (38).

Crystal violet staining. For evaluation of the overall inhibitory effect on cell number, the DU145 cells were treated with paclitaxel once and/or MSeA daily without medium change for 4 days in 6-well plates. After treatment, the culture medium was removed and the cells were fixed in 1% glutaraldehyde solution in PBS for 15 min. The fixed cells were stained with 0.02% aqueous solution of crystal violet for 30 min. After extensive washing with PBS, the retained dye in the stained cells was solubilized in 70% ethanol. The absorbance at 570 nm with the reference filter 405 nm was recorded using a microplate reader (Beckman Coulter, Inc.).

Immunoblot analyses. The cell lysate was prepared in ice-cold radioimmunoprecipitation assay buffer containing SDS and sodium deoxycholate, as described previously (38). For tumor tissue lysate preparation, the finely minced tissue pooled from five to six tumors was homogenized in ice-cold lysis buffer with a motor-driven hand-held homogenizer. The homogenate was centrifuged for 30 min at 100,000 × *g*. The supernatant fraction was recovered and immediately assayed for its protein concentration. Immunoblot analyses were essentially as described (38), except that the signals were detected by enhanced chemofluorescence with a Storm 840 scanner (Molecular Dynamics).

Reverse transcription-PCR. Total RNA was extracted using an RNeasy Mini kit (Qiagen) and was used for reverse transcription according to the manufacturer's instruction (Invitrogen). The primers used for PCR analysis were synthesized by Sigma-Genosys. The sequences of the primers were as follows: Bcl-XL, 5′-GGAGGCAGGC-GACGAGTTTGAA-3′(forward) and 5′-AAGGGGGTGGGAGGGTA-GAGTGG-3′ (reverse); survivin, 5′-GCCTGGCAGCCCTTTCTCAA-3′ (forward) and 5′-CTCGATGGCACGGCGCACTTTCT-3′ (reverse); and glyceraldehyde-3-phosphate dehydrogenase, 5′-TCA AGA AGG TGG TGA AGC AG-3′ (forward) and 5′-CTT ACT CCT TGG AGG CCA TG-3′ (reverse). The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase was used as internal control for RNA integrity and loading normalization. PCR was repeated with different number of cycles to ensure the detection of products was within the linear amplification range.

Animal study with paclitaxel/MSeA combination. The animal use protocol was approved by the Institutional Animal Care and Use Committee of the University of Minnesota, and carried out at the Hormel Institute's Association for Assessment and Accreditation of Laboratory Animal Care–accredited animal facility. Briefly, 2×10^6 DU145 cells were mixed with Matrigel (Becton Dickinson) and injected subcutaneously into the right flank of 7-week-old male BALB/c athymic

Figure 1. MSeA enhances induction of caspase-mediated apoptosis by paclitaxel (Taxol) or docetaxel in androgen-independent prostate cancer cells *in vitro. A,* apoptosis detected by Annexin V staining of DU145 cells after 24 h treatment with MSeA, Taxol, or their combination (*combo*). Combination induces a significant increase of apoptosis compared with other treatments (ANOVA, $P < 0.001$). *B,* synergistic suppression of DU145 cell number after 4 d of daily MSeA treatment with a single Taxol treatment at time 0. After treatments, cells were fixed with glutaraldehyde and stained with 0.02% aqueous solution of crystal violet and photographed (shown here a typical 6-well plate). The stained cells were solubilized with 70% ethanol, and the absorbance was evaluated using a plate reader (bar graph, $n = 3$ wells). *C,* apoptosis estimated by death ELISA assay in PC-3 cells after 24 and 48 h treatments with MSeA, Taxol, or their combination ($n = 4$). The data for 24 and 48 h treatments were evaluated separately. *D,* Western Blot detection of cleaved PARP and caspase-3 in PC-3 (*left*) and DU145 cells (*right*) after 24 h treatment. *E,* apoptosis estimated by death ELISA assay in DU145 and PC-3 cells after 24 h treatments with MSeA, docetaxel, or their combination ($n = 4$). Columns, mean; bars, SE. Data bars sharing the same letter were not statistically different ($P > 0.05$).
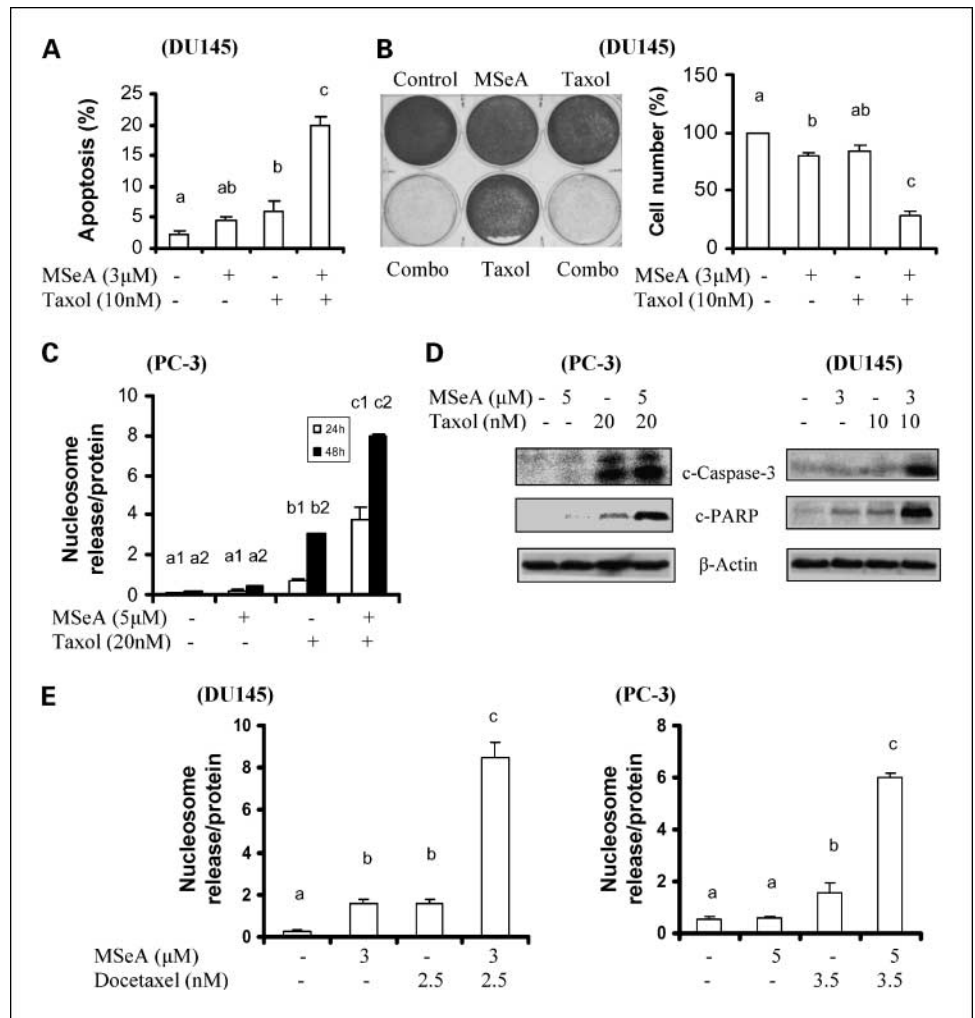
nude mice (NxGen BioSciences). The mice were fed a commercial rodent chow. Six groups of 11 mice each were used as follows: group 1, control; group 2, MSeA (2 mg selenium per kg body weight); group 3, paclitaxel (5 mg per kg body weight); group 4, paclitaxel (10 mg per kg body weight); group 5, MSeA combining with paclitaxel (5 mg per kg body weight); and group 6, MSeA combining with paclitaxel (2.5 mg per body weight). One day after cancer cell inoculation, mice in groups 1, 3, and 4 were given a daily oral treatment of water (vehicle). Mice in groups 2, 5, and 6 were given a daily oral treatment of MSeA (2 mg selenium per kg body weight). Paclitaxel injection was given once per week for 6 weeks, starting 8 days after cancer cell inoculation. Tumors were measured twice per week with a caliper, and tumor volume was calculated using the following formula: $1/2 (w_1 \times w_2 \times w_2)$, where w_1 represents the larger tumor diameter, and w_2 represents the smaller tumor diameter. We chose paclitaxel as a representative taxane drug for the *in vivo* study because it had been extensively studied by others and was more readily available than docetaxel.

Tumor tissue selenium content and pharmacokinetics of orally delivered MSeA. We saved DU145 xenograft tissues from one experiment after daily oral dosing of MSeA for 7 weeks and analyzed the selenium content. Because little data existed concerning the serum selenium response profile to MSeA in mice, we determined the pharmacokinetics of MSeA in nude mice after a single oral dose at 3 mg selenium/kg body weight at the following time points: 1, 3, 6, 12, and 24 h. Four mice were killed at each time point for blood and tissue collection. Serum samples were obtained and stored at -70°C until analyzed, each in

duplicate. Selenium content in serum and tumor xenograft was determined by using acid-wet digestion and analysis by hydride generation, atomic absorption spectrometry at Grand Forks Human Nutrition Center.

Transient transfection. DU145 cells were transiently transfected with pcDNA3.1-survivin plasmid (generously provided by Dr. Dario C. Altieri, University of Massachusetts) or control plasmid with the use of Q3 Lipofectamine 2000 (Invitrogen). To monitor the transfection efficiency, cells were transfected in parallel with green fluorescent protein plasmids (pGFP-N1; CLONTECH). The transfection efficiency was ~ 60% to 70%.

Statistical analysis. Data are presented as mean ± SE unless noted otherwise. ANOVA were done on data set with multiple groups with *post hoc* comparison of means. Statistical difference was set at *P* values of <0.05.

Results

MSeA enhanced apoptosis induced by taxane drugs paclitaxel and docetaxel in DU145 and PC-3 cells. Our previous work has shown that MSeA enhanced paclitaxel-induced apoptosis of DU145 cells, in which apoptosis was measured by a cell death ELISA assay for oligonucleosomal fragments (34). In the current study, we verified the increased apoptosis through detecting the Annexin V-binding of externalized phosphatidyl-
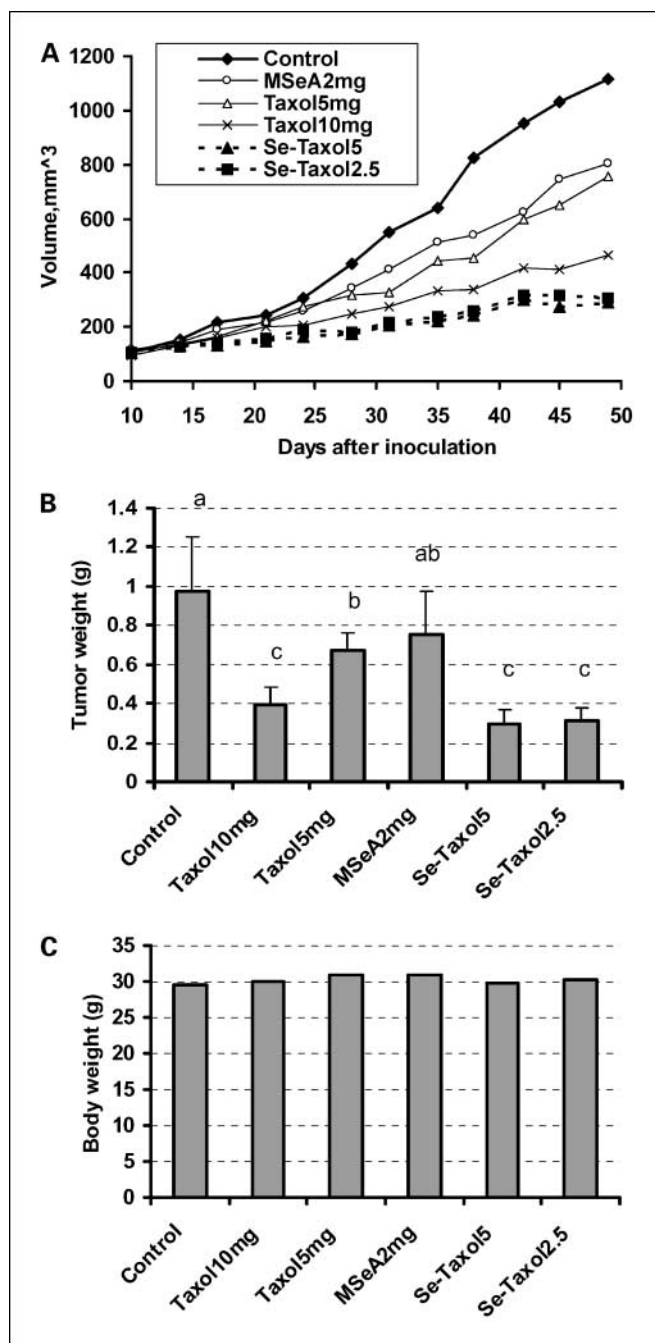
Figure 2. Synergistic suppression of DU145 xenograft tumor growth by Taxol/MSeA combination in nude mice *in vivo. A,* tumor growth kinetics; the mean calculated tumor volume was plotted without error bars for clarity. *N* = 11 mice per group. Daily MSeA oral treatment was started 1 d after tumor inoculation. Weekly Taxol i.p. injection was started 8 d after tumor inoculation. *B,* final tumor weight at termination of experiment (52 d after tumor inoculation). *N* = 11 mice per group. Columns, mean; bars, SE. Data bars sharing the same letter were not statistically different (*P* > 0.05). *C,* body weight of mice at termination of experiment. There was no statistical difference among all groups.

inhibitory effect of the combination on cell growth and survival over a 4-day duration was determined as described in Materials and Methods. As shown in Fig. 1*B*, daily addition of MSeA or a one-time paclitaxel treatment each alone caused ∼20% reduction of cell number compared with control cells, whereas >70% reduction of cell number was achieved by the combined treatment. Taken together, these data support the enhancement effect of MSeA on paclitaxel-induced apoptosis in DU145 cells.

Increased PI3K-AKT activity through PTEN-inactivation is often associated with aggressive cancer (39). In the PTEN-null HRPCa PC-3 cells (40), MSeA and paclitaxel also induced synergistic enhancement of apoptosis at both 24 and 48 h (Fig. 1*C*). The enhanced apoptotic effects of the combination treatment correlated with greater caspase-3 activation and PARP cleavage in both PC-3 (Fig. 1*D, left*) and DU145 cells (Fig. 1*D, right*). Moreover, the apoptosis enhancement action of MSeA was applicable to docetaxel, which is clinically indicated for HRPCa treatment, in both DU145 and PC-3 cells (Fig. 1*E*). Collectively, these results show that MSeA increased taxane-induced apoptosis in both DU145 (wild-type PTEN, low AKT) and PC-3 HRPCa cells in a more than additive manner, irrespective of their AKT/PTEN-status, and suggest that MSeA might enhance the therapeutic efficacy of taxanes *in vivo* against HRPCa.

MSeA and paclitaxel combination suppressed tumor growth in vivo. The impressive results from the *in vitro* studies prompted us to examine this prediction *in vivo* in the DU145 xenograft model in athymic nude mice. As shown in Fig. 2*A* and *B*, weekly treatments with paclitaxel (5 and 10 mg/kg) led F2 to a dose-dependent inhibition of tumor growth (ANOVA, *P* < 0.01), with 10 mg/kg dose decreasing the final tumor weight by 60%. The effect of daily oral treatment with MSeA was not statistically significant from the control, although a 23% numerical reduction of final tumor weight was noted. Combining MSeA with 5 mg/kg of paclitaxel led to a 70% reduction of final tumor weight. Even more impressive was that MSeA combined with 2.5 mg paclitaxel/kg body weight resulted in a 68% reduction of the final tumor weight, as effective as a 4-fold dosage of paclitaxel (10 mg/kg body weight). No treatment group showed decreased body weight (Fig. 2*C*), indicating the combination did not result in additive toxicity and was well-tolerated by the mice. These results support the *in vivo* synergistic inhibition of human prostate cancer xenograft growth by the combination regimen.

Bcl-XL and survivin as potential molecular targets of MSeA in vivo. To understand the mechanistic basis for the enhancement effects, we first established that daily oral dosing of MSeA for 7 weeks resulted in a dose-dependent increase of tumor selenium content (Fig. 3*A*). In a separate experiment, we F3 determined the acute pharmacokinetic response after a single dose of 3 mg/kg. Serum selenium reached a peak level of ∼12.5 µmol/L at 1 h and declined afterward with a half clearance of $t_{1/2}$ = 8 h, returning to the basal level of ∼6 µmol/L by 24 h (Fig. 3*B*). The peak level corresponded to a maximal serum-achievable increment of selenium of 6.5 µmol/L with the 3 mg/kg dose. The results support the relevance of the *in vitro* exposure levels evaluated in the cell culture experiments in Fig. 1 and in further studies (Figs. 4–6). F4F5F6

It has been shown that Bcl-XL and survivin play important roles for mediating paclitaxel resistance in a number of cell types including prostate cancer (17, 23, 29). We hypothesized

F1 serine by flow cytometry (Fig. 1*A*). The MSeA and paclitaxel treatment for 24 h each caused minimal apoptosis (1.1- and 1.8-fold over the control cells, respectively). Combining MSeA with paclitaxel induced apoptosis by 8.1-fold above the control. The increase of apoptosis by the combination was ∼3-fold higher than the sum of each treatment alone. The overall

Figure 3. *In vivo* selenium status and regulation of Bcl-XL and survivin in DU145 xenograft tumors by MSeA treatment of nude mice. *A,* tumor selenium content after daily oral dosing of MSeA (1 and 3 mg selenium per kg body weight) for 7 wk; *n* = 8-10 tumors per group). Columns, mean; bars, SE. Data bars sharing the same letter were not statistically different (*P* > 0.05). *B,* pharmacokinetics of an oral dose (3 mg selenium/kg) of MSeA in nude mice. At each time point, four mice were used for blood collection and sacrificed. Serum selenium was measured. Points, mean *n* = 4; bars, SE. *C,* dose-dependent down-regulation of Bcl-XL and survivin *in vivo* after daily oral dosing of MSeA (1 mg and 3 mg selenium per kg body weight) for 7 wk. *D,* inhibition of paclitaxel-induced Bcl-XL and survivin in xenograft DU145 tumors by MSeA treatment of nude mice for 52 d. For *C* and *D*, equal portions of five to six tumors from each group were pooled for protein lysate preparation for Western blot detection.
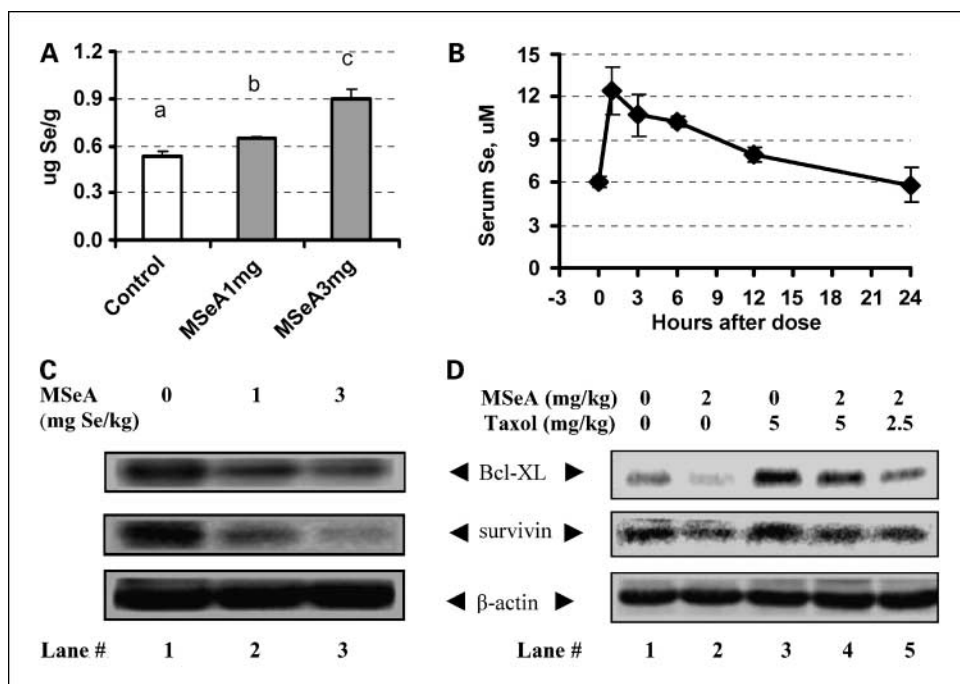
that regulation of these apoptosis/caspase regulatory proteins by MSeA might be involved in enhancing caspase activation and, hence, the *in vivo* efficacy. As shown in Fig. 3*C*, MSeA oral treatment for 7 weeks (same experiment as Fig. 3*A*) decreased the expression of Bcl-XL and survivin proteins in the xenograft tumors in a dose-dependent manner. In the paclitaxel/MSeA experiment described in Fig. 2, down-regulation of these proteins was observed in MSeA-treated tumor samples (Fig. 3*D*, *lane 2* versus *1*), whereas paclitaxel treatment alone significantly increased the abundance of these proteins (Fig. 3*D*, *lane 3* versus *1*). MSeA attenuated the paclitaxel-induced expression of Bcl-XL and survivin proteins (*lanes 4* and *5* versus *3*). Our data support the down-regulation by MSeA of both Bcl-XL and survivin as potential targets of MSeA to increase caspase activation and apoptosis execution induced by paclitaxel to enhance its *in vivo* therapeutic efficacy.
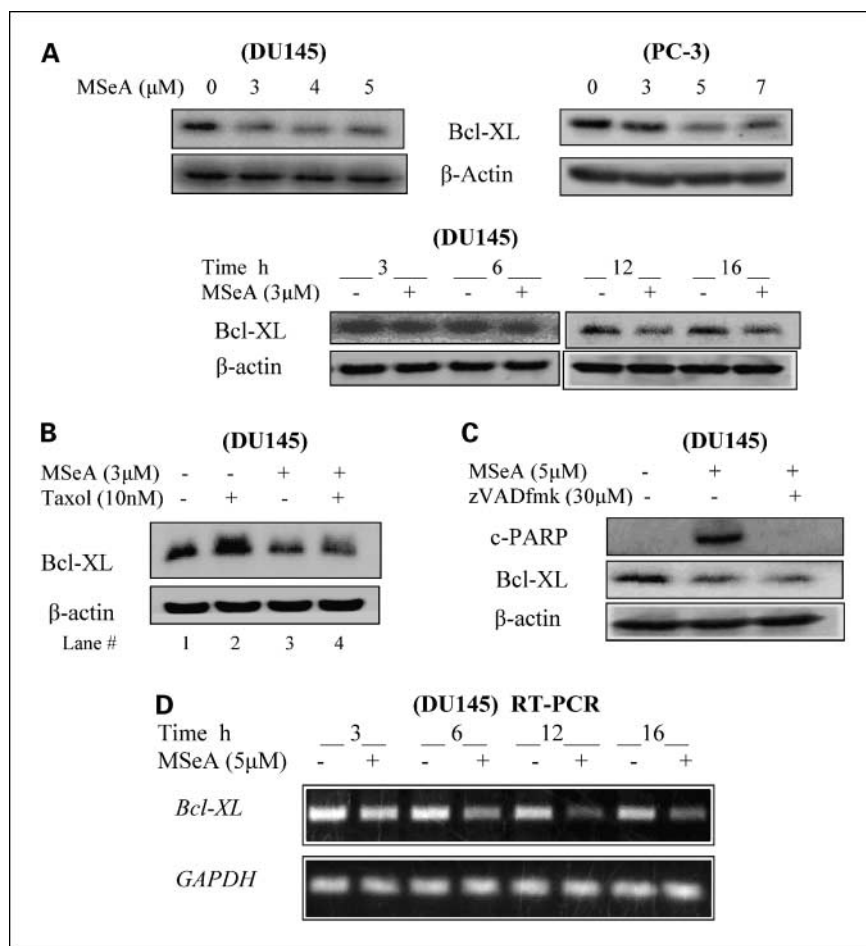
Mechanisms of down-regulation of Bcl-XL expression by MSeA. Next, we used cell culture models to delineate the mechanisms of down-regulation of Bcl-XL by MSeA. DU145 and PC-3 cells were treated with increasing concentrations of MSeA for 12 and 24 h, respectively. MSeA in the concentration range achievable in the mouse serum decreased the expression of Bcl-XL protein in a concentration-dependent manner in both DU145 and PC-3 cells (Fig. 4*A*). A time course experiment in DU145 cells (Fig. 4*A*) showed that down-regulation of Bcl-XL by MSeA occurred at 12 h of treatment. Paclitaxel increased Bcl-XL abundance (Fig. 4*B*, *lane 2* versus *1*) and the paclitaxel-induced expression of Bcl-XL was diminished by MSeA (Fig. 4*B*, *lane 4* versus *2*). These *in vitro* results were consistent with the *in vivo* data (Fig. 3*C* and *D*). The cell culture model therefore provided a reasonable system to address the mechanisms of Bcl-XL down-regulation by MSeA.

Because down-regulation of Bcl-XL by MSeA occurred in a similar time frame as caspase activation in DU145 cells (38), and Bcl-XL has been shown as a target of caspase-8 (41), we asked whether decreased Bcl-XL abundance was a consequence

of caspase activation. As shown in Fig. 4*C*, treatment with 30 μmol/L pan-caspase inhibitor completely blocked caspase-mediated PARP cleavage and apoptosis, but failed to prevent the decrease of Bcl-XL by MSeA, ruling out this possibility. On the other hand, treatment with MSeA caused a time-dependent decrease of Bcl-XL mRNA level as early as 6 h (Fig. 4*D*), preceding its protein change by several hours (Fig. 4*A*). Therefore, a transcriptional mechanism was most likely involved in the down-regulation of this protein by MSeA.

Mechanisms of the down-regulation of survivin by MSeA. Taking a similar approach as the analyses of Bcl-xL, we show that MSeA decreased the expression of survivin in a concentration-dependent manner in DU145 and PC-3 cells (Fig. 5*A*). Time course studies showed that MSeA caused a rapid (as early as 6 h) and persistent down-regulation of survivin expression in PC-3 cells (Fig. 5*A*). Paclitaxel treatment alone induced the expression of survivin protein in both DU145 and PC-3 cells, whereas MSeA abolished the paclitaxel-induced survivin expression (Fig. 5*B*).

We ruled out caspase activation as a cause of survivin down-regulation by MSeA as supported by the lack of recovery of survivin abundance after caspase-mediated PARP cleavage was blocked (Fig. 5*C*). Because survivin has a short half-life (42) and degradation through the proteasomal pathway is a well-known mechanism of regulation of its protein abundance (43), we hypothesized that down-regulation of survivin by MSeA was at least in part through promoting proteasome-mediated degradation. To test this, we blocked the proteasomal pathway with the inhibitor MG-132, which resulted in an expected increase of survivin protein (Fig. 5*D*, *lane 3* versus *1*). Whereas treatment with MSeA led to a decrease of survivin (Fig. 5*D*, *lane 2* versus *1*), combining MG-132 with MSeA completely prevented the suppression effect of MSeA (*lane 4* versus *2*). Reverse transcription-PCR analysis of survivin mRNA revealed that MSeA treatment decreased survivin transcript level after 6 h of exposure in DU145 (Fig. 5*E*). A similar modest effect on survivin mRNA level by MSeA was also seen in PC-3 cells (data not shown).

Figure 4. Mechanistic studies of down-regulation of Bcl-XL by MSeA in cell culture. *A,* dose-dependent down-regulation of Bcl-XL (detected by immunoblot) by MSeA in DU145 (12 h) and PC-3 cells (24 h), and time course of down-regulation of Bcl-XL by MSeA in DU145 cells. *B,* MSeA attenuation of Bcl-XL induction (detected by immunoblot) by paclitaxel in DU145 cells (12 h). *C,* lack of effect of a general caspase inhibitor on MSeA to down-regulate Bcl-XL (detected by immunoblot) in DU145 cells (16 h). *D,* time course of inhibition of Bcl-XL mRNA transcript (detected by reverse transcription-PCR) by MSeA in DU145 cells.

Together, these data support both proteasomal degradation of survivin protein and suppression of its mRNA transcript level as major mechanisms of its down-regulation by MSeA.

Effect of overexpression of Bcl-XL and survivin on MSeA/ paclitaxel-induced apoptosis. To address the functional significance of the down-regulation of Bcl-XL by MSeA in MSeA/ paclitaxel-induced apoptosis, we examined the effect of overexpressed Bcl-XL in stable-transfectant DU145 cells. The expression level of Bcl-XL was validated by Western blot analysis (Fig. 6*A*). MSeA/paclitaxel-induced apoptosis was significantly attenuated in DU145-Bcl-XL cells compared with the vector control cells (Fig. 6*B*). Next, we tested the effect of survivin through transient transfection of DU145 cells with a wild-type survivin construct. The transfection efficiency estimated by green fluorescent protein plasmid was 60% to 70%. The enforced survivin expression was validated by Western blot (Fig. 6*C*). As shown in Fig. 6*D*, ectopic expression of survivin led to a significant attenuation of apoptosis induced by MSeA/ paclitaxel in comparison with the vector control cells. These results support the hypothesis that down-regulation of Bcl-XL and survivin by MSeA enhances apoptosis induction by paclitaxel by lowering the threshold of caspase activation.

Discussion

Taxane-based combination chemotherapy is the only regimen approved by the Food and Drug Administration for treatment of HRPCa with very modest survival benefit. However, development of drug resistance and dose-limiting toxicity pose major clinical challenges (1). Our *in vitro* and *in vivo* data suggest a mechanism-based combination approach for improving taxane drug efficacy in HRPCa therapy as well as for potentially lowering the toxic side effects of taxane drugs through reduced drug dosage.

As alluded to in the Introduction, overexpression of antiapoptotic proteins in cancer cells has been recognized as a novel mechanism of developing resistance to anticancer drugs including taxanes. The significance of the two-pronged action of MSeA to down-regulate both Bcl-XL and survivin reported here deserves further elaboration. The majority of human cancers has been found to overexpress the Bcl-2 family of antiapoptotic proteins, mainly Bcl-XL and Bcl-2 (13–19). Whereas cancers with high expression of Bcl-XL/ Bcl-2 proteins are resistant to a variety of chemotherapeutic agents, a small molecular chemical inhibitor of these proteins was found to induce the regression of solid tumors and to potentiate the activity of cytotoxic drugs (18, 19). Given the extensive evidence that Bcl-XL plays a critical role in cancer cell survival and chemoresistance, development of compounds that either diminish Bcl-XL protein levels or inhibit its function has been a focus of extensive research. This approach has shown very good preclinical promise as shown by the cited example (18, 19). Paradoxically, induction of Bcl-XL by microtubule targeting chemotherapeutic drugs has

been found in a number of cancer types (10, 11), perhaps as a compensatory survival response. Inhibition of Bcl-XL either by genetic approaches or by small molecule inhibitors has been consistently shown to sensitize cancer cells to taxane drugs (17–19).

Survivin, the newest and the best-studied member of inhibitors of apoptosis proteins, is highly expressed in most human cancers but undetectable in most normal adult tissues (27, 44). It intersects multiple signaling networks implicated in the inhibition of apoptosis, cell proliferation, angiogenesis, and cellular stress response (27, 45). Because of its unique role in multiple cellular networks, it is no surprise that survivin has become an attractive target for cancer drug design. Inhibition of survivin either by RNAi, by antisense, or by dominant-negative mutant sensitizes cancer cells to paclitaxel (22, 23, 46), whereas overexpression of the wild-type survivin in cancer cells increases their resistance to paclitaxel, compromising drug efficacy (23). Induction of survivin by paclitaxel has been reported in a variety of cancer types including prostate cancer cells (22, 23). Therefore, targeting survivin is a rational approach to overcome paclitaxel resistance.

In the present study, we provided *in vivo* and *in vitro* evidence of the ability of MSeA to target both proteins. We observed an increased Bcl-XL abundance and a possible increase of phosphorylation (retarded migration on gel; Fig. 4*B*, *lane 2*

versus *1*) in paclitaxel-treated HRPCa cells and an increase in its abundance in paclitaxel-treated tumor tissue (Fig. 3*D*, *lane 3* versus *1*). Combining with MSeA abolished or significantly attenuated the paclitaxel-induced changes of Bcl-XL both *in vitro* and *in vivo*. Down-regulation of Bcl-XL by MSeA correlated with enhanced caspase-3 activation in paclitaxel/MSeA-combination treatment (Fig. 1*D*). Regarding the mechanisms of down-regulation of Bcl-XL protein by MSeA, our data strongly supported a rapid decrease of mRNA, a transcriptional mechanism playing a major role (Fig. 4*D*), and ruled out caspase cleavage (Fig. 4*C*) as a contributing cause. The fact Q4 that DU145 cells with overexpressed Bcl-XL significantly diminished paclitaxel/MSeA-induced apoptosis (Fig. 6*A* and *B*) provided further support for a functional role of down-regulation of Bcl-XL by MSeA to enhance paclitaxel-induced apoptosis signaling.

As for survivin, we found that MSeA not only decreased the basal level of survivin (Fig. 5) but also inhibited the paclitaxel-induced survivin expression in DU145 and PC-3 cells (Fig. 5) and in DU145 xenograft in nude mice (Fig. 3). Mechanistically, proteasomal protein degradation played a major role in MSeA-induced down-regulation of survivin, whereas transcriptional regulation also contributed to some extent (Fig. 5). Our results agreed with and extended a recently published mechanistic study that showed an inhibition of survivin

Figure 5. Mechanisms of down-regulation of survivin by MSeA in cultured cells. *A,* dose-dependent down-regulation of survivin (detected by immunoblot) by MSeA in DU145 (12 h) and PC-3 cells (24 h), and time course of down-regulation of survivin by MSeA in PC-3 cells. *B,* MSeA effect on paclitaxel-induced survivin (detected by immunoblot) in DU145 (16 h) and PC-3 cells (8 h). *C,* lack of effect of caspase inhibition on down-regulation of survivin by MSeA in DU145 cells (16 h). *D,* effect of the proteasomal inhibitor MG-132 on down-regulation of survivin (detected by immunoblot) by MSeA in DU145 cells (12 h). *E,* time course of effects of MSeA on survivin mRNA transcript (detected by reverse transcription-PCR) in DU145 cells.
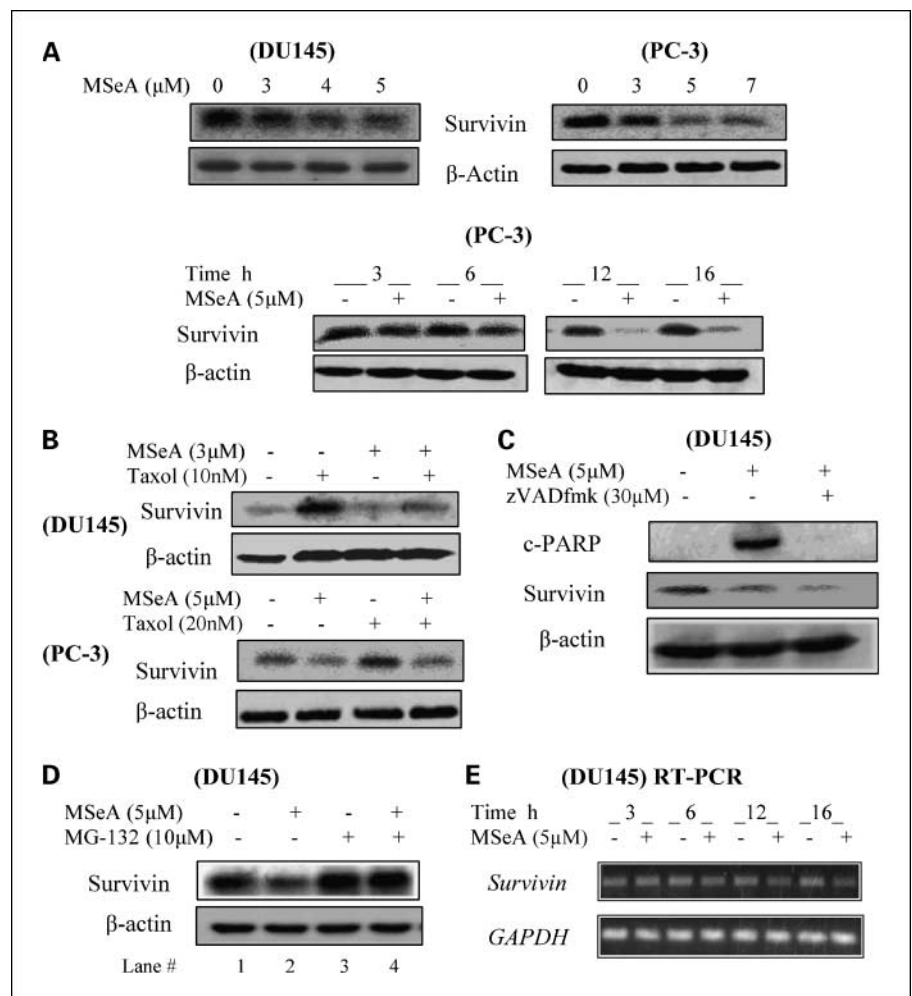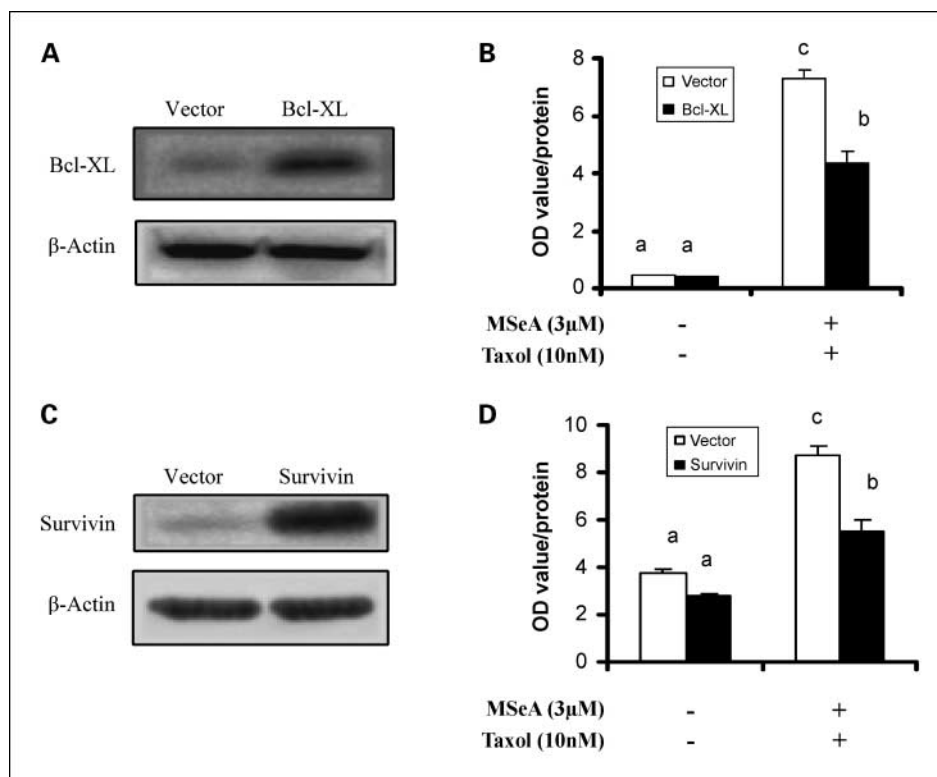
Figure 6. Impact of up-regulation of Bcl-XL or survivin on MSeA/paclitaxel-induced apoptosis in DU145 cells. *A,* immunoblot analysis of Bcl-XL expression in Bcl-XL stable transfectant and vector transfectant cells. *B,* apoptosis (estimated by Cell Death ELISA) induced by MSeA/paclitaxel combination in DU145 cells overexpressing Bcl-XL and in vector control cells (24 h, $n = 4$). Columns, mean; bars, SD. Data bars sharing the same letter were not statistically different ($P > 0.05$). *C,* immunoblot analysis of survivin expression in DU145 cells after transient transfection of wild-type survivin or vector control. The transfection efficiency (~ 60-70%) was monitored by transfection of green fluorescent protein plasmid, and the cells were harvested 24 posttransfection. *D,* apoptosis induction (estimated by Cell Death ELISA) by MSeA/paclitaxel in DU145 cells with transient expression of survivin or vector plasmid. The treatment was started 24 h posttransfection for an additional 24 h ($n = 4$). Columns, mean; bars, SD. Data bars sharing the same letter were not statistically different ($P > 0.05$).

promoter transcription by MSeA through targeting Sp-1 binding (47).

In summary, combining serum-achievable levels of MSeA with taxane drugs resulted in greater than additive suppression of HRPCa cell growth and survival *in vitro*. The simultaneous down-regulation by MSeA of both Bcl-XL and survivin, in spite of some mechanistic subtleties, blunted or abolished the induction of these proteins by taxanes, permitting the augmented activation of caspases and apoptosis execution. These actions may, to some extent, account for the synergistic enhancement of growth inhibitory efficacy of Taxol by MSeA *in vivo*. Our data suggest that MSeA holds promising therapeutic

potential as a novel agent to combine with taxane drugs to improve chemotherapy of HRPCa.

Acknowledgments

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